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Thiopurine S-Methyltransferase Deficiency: Two Nucleotide Transitions Define the Most Prevalent Mutant Allele Associated with Loss of Catalytic Activity in Caucasians

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Summary

The autosomal recessive trait of thiopurine S-methyltransferase (TPMT) deficiency is associated with severe hematopoietic toxicity when patients are treated with standard doses of mercaptopurine, azathioprine, or thioguanine. To define the molecular mechanism of this genetic polymorphism, we cloned and characterized the cDNA of a TPMT-deficient patient, which revealed a novel mutant allele (*TPMT*3*) containing two nucleotide transitions ($G^{460}\rightarrow A$ and $A^{719}\rightarrow G$) producing amino acid changes at codons 154 (Ala→Thr) and 240 (Tyr→Cys), differing from the rare mutant *TPMT* allele we previously identified (i.e., *TPMT*2* with only $G^{238}\rightarrow C$). Site-directed mutagenesis and heterologous expression established that either *TPMT*3* mutation alone leads to a reduction in catalytic activity ($G^{460}\rightarrow A$, ninefold reduction; $A^{719}\rightarrow G$, 1.4-fold reduction), while the presence of both mutations leads to complete loss of activity. Using mutation specific PCR-RFLP analysis, the *TPMT*3* allele was detected in genomic DNA from ≈75% of unrelated white subjects with heterozygous phenotypes, indicating that *TPMT*3* is the most prevalent mutant allele associated with TPMT-deficiency in Caucasians.

Introduction

Thiopurine methyltransferase (TPMT, E.C.2.1.1.67) is a cytoplasmic enzyme that preferentially catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds, including the anticancer agents 6-mercaptopurine (6MP) and 6-thioguanine, and the immunosuppressant azathioprine. TPMT activity exhibits genetic polymorphism, with ~89% of Caucasians and African-Americans having high TPMT activity, 11% intermediate activity (presumed heterozygotes), and ~1 in 300 inheriting TPMT deficiency as an autosomal recessive trait (Weinshilboum and Sladek 1980; McLeod et al. 1994). TPMT activity is typically measured in erythrocytes, as the level of TPMT activity in human liver, kidney, and leukemic lymphoblasts correlates with that in erythrocytes (Van Loon and Weinshilboum 1982; Szumlanski et al. 1992; McLeod et al. 1995*b*).

Mercaptopurine, thioguanine, and azathioprine are prodrugs with no intrinsic activity, requiring intracellular conversion to thioguanine nucleotides (TGN), with subsequent incorporation into DNA, as one mechanism of their antiproliferative effects (Elion 1989). Alternatively, these drugs are metabolized to 6-methyl-mercaptopurine (MeMP) or 6-methyl-thioguanine (MeTG) by TPMT or to 6-thiouric acid (6TU) by xanthine oxidase; MeMP, MeTG, and 6TU are inactive metabolites. Thus, metabolism of thiopurines by TPMT shunts drug away from the TGN activation pathway. Clinical studies with 6MP and azathioprine have established an inverse correlation between erythrocyte TPMT activity and erythrocyte TGN accumulation; patients who less efficiently methylate these thiopurines have more extensive conversion to TGN (Lennard et al. 1989, 1990). Moreover, patients with TPMT deficiency accumulate significantly higher erythrocyte TGN if treated with standard dosages of 6MP or azathioprine, leading to potentially fatal hematopoietic toxicity, unless the thiopurine dosage is lowered substantially (e.g., 8–15-fold reduction) (Evans et al. 1991; Lennard et al. 1993; McLeod et al. 1993; Schutz et al. 1993). The majority of such patients are identified only after experiencing severe toxicity, even though prospective measurement of erythrocyte TPMT activity has been advocated by some (Lennard et al. 1987). Unfortunately, TPMT assays are not widely available, and newly diagnosed leukemia patients and organ transplant recipients are frequently given erythrocyte transfusions, precluding measurement of their constitutive TPMT activity before thiopurine therapy is initiated. Alternatively, if the inactivating mutations of the

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human *TPMT* gene can be identified, PCR-based methods can be developed to determine TPMT genotype and prospectively predict phenotype, as is now possible for such drug-metabolizing enzymes as debrisoquin-hydroxylase (Heim et al. 1990) and N-acetyltransferase (Dupret et al. 1994).

Isolation and characterization of a human cDNA encoding TPMT activity (Honchel et al. 1993) and the subsequent identification of a single point mutation leading to loss of TPMT catalytic activity (Krynetski et al. 1995*b*) have provided initial insights into the molecular basis of this genetic polymorphism. However, the mutant TPMT allele with the $G^{238}\rightarrow C$ transversion (*TPMT**2) is not present in the majority of TPMT-deficient individuals or those with heterozygous phenotypes, leaving uncertainty about the most common molecular defect underlying this inherited trait. Identification of the predominant *TPMT* mutations would not only offer a DNA-based strategy for prospectively identifying heterozygotes and TPMT-deficient patients, prior to treatment with potentially toxic dosages of thiopurines, it would also more completely define the genetic basis for this polymorphism. To this end, we report the identification and characterization of the predominant mutant allele associated with TPMT-deficiency in Caucasians.

Material and Methods

Cloning of TPMT cDNA

After informed written consent from the parent for a study protocol approved by the institutional review board, total RNA was isolated (Chomczynski and Sacchi 1987) from normal leukocytes of a 5-year-old boy with acute lymphocytic leukemia in complete remission. This child had developed severe hematopoietic toxicity on standard dosages of 6MP (75 mg/m^2/d). At the initial presentation of toxicity, his erythrocyte concentration of TGN was >15-fold higher than the population median (4,400 versus 280 pmol/ml packed red blood cells [pRBC]). Subsequently, he was documented to have TPMT-deficiency (0.6 U/ml pRBC). First-strand cDNA was synthesized from 2 μg of total RNA and then amplified to obtain TPMT-coding region, as described elsewhere (Krynetski et al. 1995*b*). The PCR fragments were either made blunt and cloned into the *Sma*I site of plasmid pGEM-7Zf(+) (Promega), or directly cloned into PCR™II (Invitrogen). Plasmids were purified with Qiagen plasmid kits and sequenced with an automated sequencer, using the cycle sequencing reaction employing fluorescence-tagged dye terminators (PRISM, Applied Biosystems).

Site-Directed Mutagenesis of TPMT cDNA

The wild-type and mutant cDNA clones were used as templates for site-directed mutagenesis. PCR conditions were as described (Krynetski et al. 1995*b*), except that annealing temperature was changed to 50°C and 1.3 U of *Pyrococcus furiosus* DNA polymerase (Stratagene) was used. After amplification, the PCR products were ligated into pYeDP 1/8-2 yeast expression vector as described elsewhere (Krynetski et al. 1995*b*). In order to prepare cDNA containing either mutation found in this patient, two reverse primers were designed for further mutagenesis. The amplification was performed either with primers A and B (5′-cggatccaaaATGGATGGTACAAGAACTTCACTTGACATTG-3′, 1–31; and 5′-cggaattcTTACTTTTCTGTAAGTAGATATAACTTTTC-3′, 709–738; respectively), using the plasmid containing mutant cDNA as the template to generate a cDNA containing only G460A (i.e., $TPMT_{460}$), or with primers A and C (5′-cggaattcTTACTTTTCTGTAAGTAGAcATAACTTTTC-3′, 709–738), using the plasmid containing wild-type cDNA as the template to generate a cDNA containing only A719G (i.e., $TPMT_{719}$). The resultant PCR products were also ligated into the expression vector. Recombinant plasmids were constructed that contained galactose-inducible *GAL10-CYC1* promoter (Cullin and Pompon 1988), either the wild-type or mutant forms of TPMT cDNA, and a phosphoglycerate kinase (PGK) terminator. Nucleotide structures of all cDNAs were confirmed by sequencing.

Expression in Yeast Cells

TPMT cDNAs were expressed in yeast strain 2805, essentially as described elsewhere (Becker and Lundblad 1993; Krynetski et al. 1995*b*). In brief, yeast cells transformed with recombinant expression vectors or the vector without TPMT cDNA (control) were grown on galactose-containing medium for 24 h at 30°C. The cytosolic fraction was isolated and resuspended in 5 mM Tris-HCl buffer (pH 7.8) containing 1×10^5 IU/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. The concentration of protein in yeast lysates was determined using the Bio-Rad *DC* protein assay (Lowry et al. 1951). The level of expression was measured by western blot analysis.

Northern Blotting

Total yeast RNA was extracted according to the method of Schmitt et al. (1990), denatured with glyoxal and dimethyl sulfoxide (DMSO), electrophoresed on a 1.4% agarose gel as described (Sambrook et al. 1989), and transferred to a Hybond™-N+ nylon membrane (Amersham). Membranes were hybridized with the radiolabeled TPMT cDNA and subsequently reprobed with *y*18S oligonucleotide as described elsewhere (Krynetski et al. 1995*b*).

Western Blot Analysis of TPMT

SDS-polyacrylamide gel electrophoresis was carried out following the method of Laemmli (1970) by using

15% acrylamide slab gels. Proteins were then electrophoretically transferred to a nitrocellulose membrane and reacted with a polyclonal rabbit antiserum against human TPMT (McLeod et al. 1995*a*). This antibody was raised in rabbits by immunization with GST-TPMT fusion protein and purified in sequence by affinity chromatography on sepharose with immobilized GST and GST-TPMT. The signals of the western blot were visualized by enhanced chemiluminescent detection (ECL kit) following manufacturer's instructions (Amersham). TPMT content in yeast lysate expressing TPMT cDNA was estimated by this analysis by using the standard of purified GST-TPMT fusion protein treated with thrombin.

TPMT Assay and Estimation of Kinetic Parameters (V_{max} and K_m)

Erythrocyte lysates were analyzed for TPMT activity by the nonchelated radiochemical assay of Weinshilboum et al. (1978). For kinetic experiments, the enzymatic reaction of TPMT was carried out at 37°C in a 1-ml mixture containing 0.1 M Tris-HCl, pH 7.5, yeast cytosol expressing TPMT, various concentrations of 6MP (4 μM–8 mM) and S-adenosyl-L-methionine (SAM; 1 μM–6 mM), plus allopurinol (20 μM), and DTT (250 μM). These reaction conditions differ from those previously published by our lab (Krynetski et al. 1995*a*); with incubation at 37°C instead of 21.5°C, analysis of MeMP product formation instead of substrate disappearance, and the use of higher substrate concentrations in the present study. The amount of TPMT in each reaction was made constant (0.57 μg TPMT) by adjusting the amount of yeast cytosol according to TPMT protein levels detected by the western blot analysis. For kinetic studies of 6MP, 1 mM of SAM was used, whereas for studies of SAM, 2 mM of 6MP was utilized. The reaction was started by the addition of yeast cytosol or 6MP (in 10 μl of DMSO), allowed to proceed for 30 min, and stopped by the addition of 100 μl of 1 M HCl. After filtration through a Centricon-3 or -10 membrane (Amicon), 100 μl of the filtrate was injected into HPLC to measure the formation of the methylated metabolite, MeMP, using a gradient system essentially as described elsewhere (Krynetski et al. 1995*a*). Nonlinear least-squares regression was used to estimate V_{max} and K_m by fitting a Michaelis-Menten model to the nontransformed data as described elsewhere (Krynetski et al. 1995*a*).

Intrinsic Stability of Recombinant TPMT Proteins

Cytosols from yeast expressing wild-type, $TPMT_{460}$, or $TPMT_{719}$ protein were incubated in 0.1 M Tris-HCl (pH 7.5) at 37°C for 0–12 h (after 3 min equilibration from 0°C to 37°C), then assayed for TPMT activity as described above, using fixed concentrations of 2 mM 6MP and 1 mM SAM. The assay of TPMT activity was allowed to proceed for 15 min at 37°C, and MeMP was measured as described above. Total protein concentrations of yeast lysate in the incubation mixture were 0.09, 0.37, and 0.08 mg/ml for the wild type, $TPMT_{460}$, and $TPMT_{719}$, respectively, to give equal amount of TPMT in the incubation. The same assay mixture without yeast lysate served as the blank, and the background values for nonenzymatic methylation (<10%) were subtracted from all values obtained. An aliquot of sample at each time point was taken for western blot analysis. The samples at 0°C, 0 h served as controls for the blots with $TPMT_{460}$ and $TPMT_{719}$.

*RFLP Analysis to Detect TPMT*3 Mutations in cDNA*

TPMT cDNA synthesized by reverse transcription-PCR was used as template in PCR amplification with primers D and E (5′-CAGGCTTTAGCATAATTTT-CAATTCCTC-3′, 779–806; 5′-CAGAAGAACCAAT-CACCG-3′, 323-340; respectively), for 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 45 s, and elongation at 72°C for 1.5 min. The synthesized DNA fragments were digested with either *Acc*I or *Mwo*I restriction endonuclease (New England Biolabs) and analyzed by electrophoresis in 2.5% MetaPhor agarose (FMC). Digestion of wild-type DNA fragments by *Mwo*I yields two fragments (340 bp and 144 bp), while the G460A mutation eliminates the *Mwo*I restriction site, yielding one fragment of 484 bp. Alternatively, the A719G mutation adds an *Acc*I restriction site, yielding two fragments (398 bp and 86 bp) when the mutation is present, but only one fragment with the wild-type sequence. DNA ladder length markers of 123 bp (GIBCO) were used to estimate the fragments' size. For these assays, PCR-amplified fragments of the *TPMT*3* cDNA or wild-type cDNA were run as controls.

*Detection of the TPMT*3 Allele in Genomic DNA*

To detect the G460A mutation, 250 ng of patient's DNA was used as template in a PCR assay using exon 7 primer F (5′-atgtaatacgactcactataACCTGGATTAAT-GGCAAC-3′) and intron 6 primer G (5′-ATAACA-GAGTGGGGAGGCTGC-3′) in buffer A (Invitrogen) containing 60 mM tris-HCl pH 8.5, 15 mM ammonium sulfate, 1.5 mM $MgCl_2$. Amplification conditions were as follows: cycle 1, 80°C for 1 min, 94°C for 2 min. Five microliters of 10 mM dNTP were added after heating to 80°C ("hot start" protocol). The reaction proceeded at 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min for 35 cycles more, and finished with extension at 72°C for 7 min. The products of the reaction were digested with *Mwo*I and analyzed as described above. *Mwo*I digestion of wild-type DNA yielded fragments of 267 bp and 37 bp, whereas DNA containing the G460A mutation was not digested, yielding a fragment of 304 bp.

To detect the A719G mutation, 250 ng of genomic DNA was PCR-amplified using a sense primer to intron 9 (5′-TGTTGGGATTACAGGTGTGAGCCAC-3′), and an antisense primer to exon 10 (5′-CAGGCTTTAGCATAATTTTCAATTCCTC-3′) yielding a 293-bp fragment containing nt 719. The PCR-amplification protocol described for the 460 mutation was used for the 719 assay. Because the *Acc*I restriction site was introduced when the 719 mutation was present, the fragment containing the mutation was cleaved (207-bp and 86-bp fragments) when digested with *Acc*I (37°C, 2 h), while the wild-type fragment was not digested.

Data Analysis

The University of Wisconsin Genetics Computer Group software package was used to analyze sequence information (Genetics Computer Group 1991). A multiple comparison procedure utilizing two-way analysis of variance with Bonferroni adjustment was used to compare differences between kinetic parameters of the wild-type versus the mutant TPMTs expressed in yeast.

Results

Cloning and Sequencing of TPMT Alleles

First-strand cDNA was synthesized from total RNA of a TPMT-deficient patient whose erythrocyte TPMT activity and protein levels were 20–30-fold less than wild-type patients (fig. 1), and clones containing the TPMT open-reading frame were obtained from six independent PCR reactions. These clones were sequenced and revealed two distinct cDNAs, nine clones each. One sequence contained only the previously described point mutation ($G^{238}\rightarrow C$), a mutant allele designated *TPMT*2*. The other sequence (fig. 2) contained two nucleotide transitions, $G^{460}\rightarrow A$ (G460A) and $A^{719}\rightarrow G$ (A719G), leading to amino acid substitutions at codon 154 ($Ala^{154}\rightarrow Thr$) and codon 240 ($Tyr^{240}\rightarrow Cys$), designated *TPMT*3*. The equal abundance of cDNA clones for these two sequences suggests they are from two alleles of the TPMT gene expressed at comparable levels in this patient.

*Detection of TPMT*3 Mutations in cDNA of Propositus Family Members*

RFLP analysis of cDNAs revealed the presence of both *TPMT*3* mutations in the propositus and his mother (fig. 3). Because G460A eliminates the recognition site of *Mwo*I and A719G adds an *Acc*I restriction site, wild-type cDNA (control) was cut by *Mwo*I, but not by *Acc*I, whereas the deficient patient's cDNA was heterozygous with respect to these restriction sites (fig. 3), consistent with this patient having two different mutant *TPMT* alleles (i.e., *TPMT*2* and *TPMT*3*). Furthermore, the mother's *TPMT*3* restriction pattern was the same as the propositus, while the father did not have the *TPMT*3* allele, indicating that this patient inherited the *TPMT*3* allele from his mother.

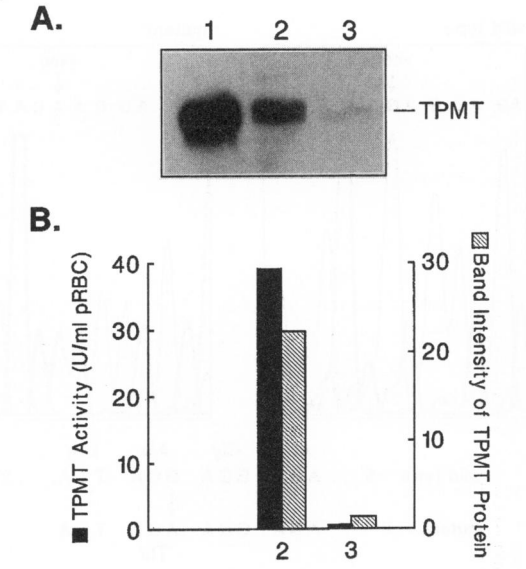

Figure 1 Relative level of TPMT protein and activity in a wild-type and a deficient patient. *A*, Western blot of RBC lysates probed by anti-TPMT antibodies. Lane 1 = wild-type TPMT cDNA expressed in yeast; lane 2 = TMPT–wild type RBC lysate; lane 3 = TPMT-deficient RBC lysate. Lysate equivalent to 2×10^6 RBC was loaded in lanes 2 and 3. Yeast lysate expressing wild-type TPMT was utilized for comparison (lane 1). *B*, Relative levels of TPMT protein and activities in erythrocytes determined by densitometry of the western blot and by the radiochemical TPMT activity assay (Weinshilboum et al. 1978), respectively.

Heterologous Expression of Wild-Type and Mutant TPMT

To determine the relative contribution of the two point mutations in *TPMT*3*, site-directed mutagenesis was used to generate mutant cDNAs with either the G460A mutation ($TPMT_{460}$) or the A719G mutation ($TPMT_{719}$). As shown in figure 4*A*, TPMT mRNA levels were similar in yeast expressing wild-type cDNA, *TPMT*3* cDNA, or the cDNAs containing either of the nucleotide transitions, suggesting that neither point mutation alone nor in combination altered transcription of TPMT cDNAs in this heterologous expression system. TPMT mRNA was not detected with yeast expressing the vector alone. In contrast, TPMT protein levels were similar between the wild-type and the $TPMT_{719}$ mutant cDNA, but protein levels for the $TPMT_{460}$ and *TPMT*3* were 4-fold and 400-fold less than the wild type, respectively (fig. 4*B*). *TPMT*3* protein was detectable only when 100-fold more of yeast lysate protein was loaded on the gel (fig. 4*B*, lane 6), whereas TPMT was not detected with yeast expressing vector without cDNA (fig. 4*B*), even with loading 150-fold more of the yeast

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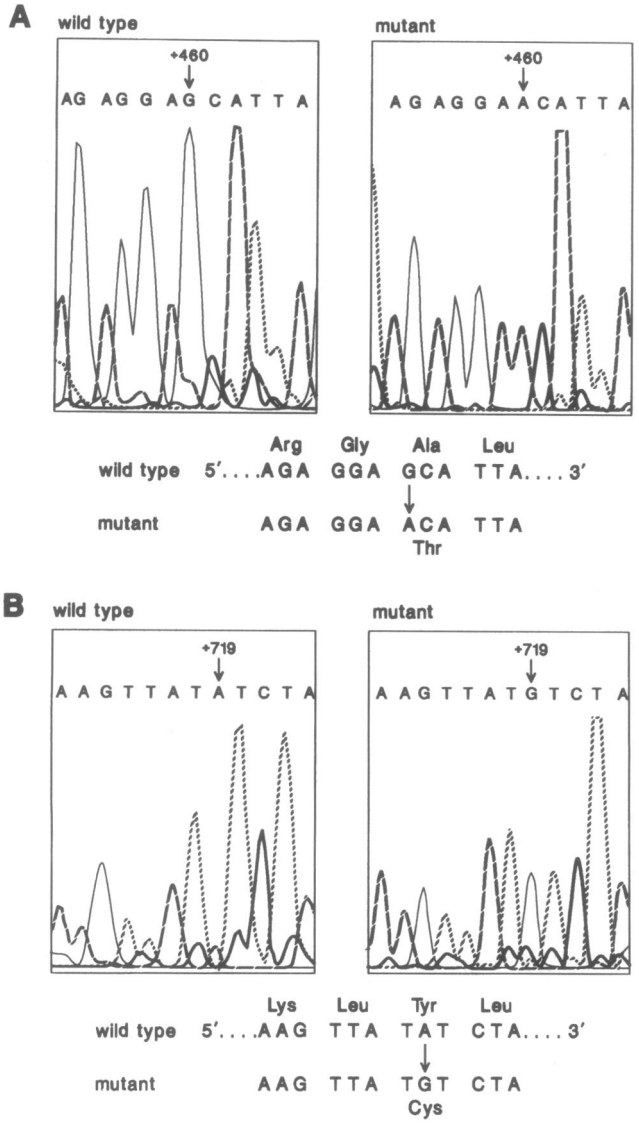

Figure 2 Differences in the wild-type and mutant TPMT cDNA sequence and deduced amino-acid sequence of the proteins encoded. Two segments of the sequences are displayed: nt 454–465 (*A*) and nt 712–723 (*B*). The adenine residue in the initiation codon is number +1.

lysate (36 μg) (data not shown). Thus, neither point mutation alone altered TPMT protein levels comparable to the cDNA with both mutations. In addition, when activity was normalized to the TPMT content in yeast lysates and compared with wild type, the catalytic activity of $TPMT_{460}$ was reduced to a greater extent (9-fold) than that of $TPMT_{719}$ (1.4-fold), whereas no activity of *TPMT*3* could be detected, even at 6MP concentrations up to 2 mM.

Michaelis-Menten Constants for S-methylation

While TPMT activity was undetectable with *TPMT*3* (thus no kinetic parameters determined), the modest activity of mutant $TPMT_{460}$ and $TPMT_{719}$ permitted estimation of kinetic parameters for both 6MP and SAM as substrates. Table 1 summarizes V_{max} and K_m values, estimated by fitting a Michaelis-Menten model to the untransformed data. All parameter estimates were significant at $P < .05$ (two-tailed *t*-test). Both K_m and V_{max} values for 6MP or SAM were significantly higher ($P < .01$) for $TPMT_{460}$ than wild type, while V_{max} and K_m for $TPMT_{719}$ were not significantly different from the wild type (table 1). Although V_{max} values of $TPMT_{460}$ for 6MP or SAM were increased (≈4-fold) compared to the wild type, the K_m for $TPMT_{460}$ was substantially greater than wild type (≈46-fold with 6MP, ≈208-fold with SAM), such that the intrinsic clearance of 6MP and SAM (i.e., V_{max}/K_m) for $TPMT_{460}$ was 13-fold and 51-fold lower than wild

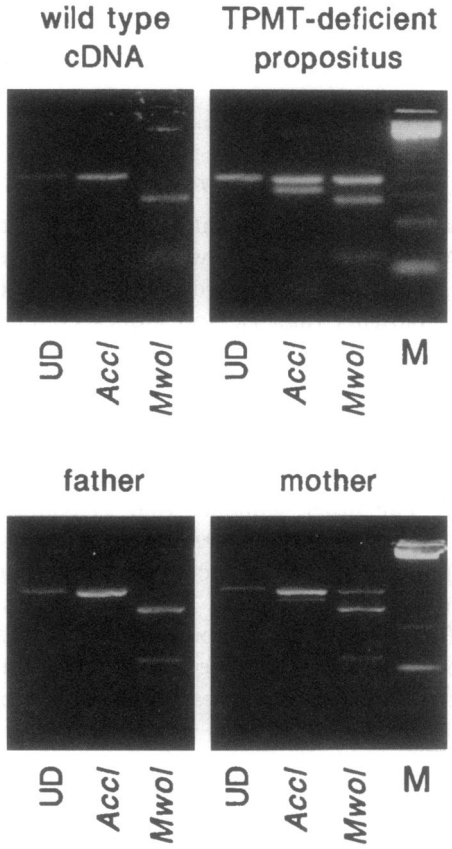

Figure 3 PCR-RFLP analysis of wild-type cDNA and cDNAs from the TPMT-deficient patient and his parents. Three samples were run for each cDNA; lanes labeled "UD" are undigested PCR-amplified cDNA fragment (nt 323–806, 484 bp); lanes labeled *Acc*I are PCR products digested by *Acc*I to yield a 484-bp fragment when the wild-type sequence was present at nt 719 or 398-bp and 86-bp fragments when the A719G mutation was present; lanes labeled *Mwo*I are PCR products digested by *Mwo*I to yield 340-bp and 144-bp fragments when the wild-type sequence was present at nt 460 or one fragment of 484 bp when the G460A mutation was present. Lanes M represent a 123-bp DNA ladder, used as markers.

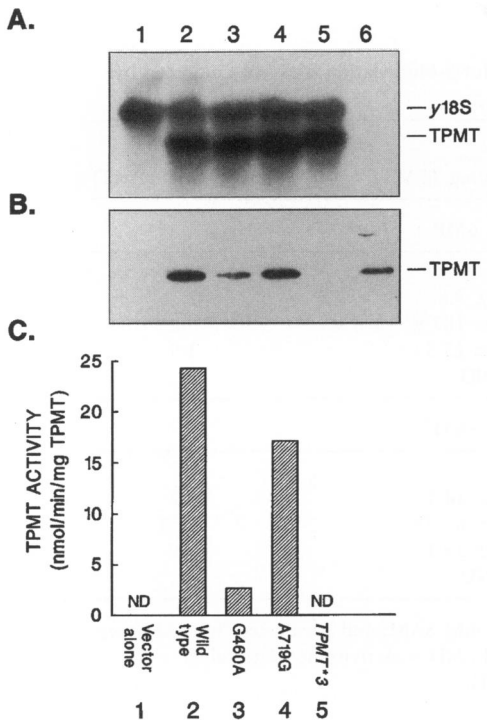

Figure 4 Comparison of TPMT mRNA (*A*, northern blot), protein (*B*, western blot), and activity levels (*C*) in yeast transformed with vector alone without any insert (lane 1), vector with wild-type cDNA (lane 2), vector with cDNA containing G460A (lane 3), vector with cDNA containing A719G (lane 4), and vector with cDNA containing both G460A and A719G (*TPMT*3*) (lane 5). *A*, Northern blot of yeast total RNA (8.4 μg) hybridized with TPMT cDNA probe and reprobed with *y*18S rRNA-specific oligonucleotide. *B*, Western blot of yeast lysates (0.25 μg for lanes 1–5, 25 μg for lane 6) with anti-TPMT antibodies, *C*, TPMT activities measured by in vitro incubation of yeast lysates with substrate concentrations of 10 μM 6MP and 1 mM SAM. ND = activity not detectable. Lane 6 of western blot depicts results when 100-fold greater protein was used from lysate of yeast transformed with *TPMT*3*.

type, respectively, while $TPMT_{719}$ was comparable to wild type.

Stability of Recombinant TPMT Proteins in Vitro

As shown in figure *5A*, both $TPMT_{460}$ and $TPMT_{719}$ proteins were rapidly inactivated at 37°C (within 4 h), while 30% of the wild-type TPMT activity remained at 12 h under the same conditions. Western blot analysis (fig. *5B–D*) shows that under these conditions TPMT protein content did not change substantially for the wild type or mutants, suggesting that the reduction in activity for these mutants largely reflects *intrinsic* instability, not degradation of the protein. In contrast, when the cDNA with both mutations (i.e., *TPMT*3*) was expressed in yeast, there was markedly (i.e., 400-fold) lower TPMT protein on western blots (fig. *4B*, lane 5) despite comparable mRNA levels, suggesting that *TPMT*3* is poorly translated or encodes an unstable protein in yeast. It is interesting that a higher-molecular-weight protein band (≈52 kD) recognized by anti-TPMT antibody, increased in a time-dependent manner with the $TPMT_{460}$ and $TPMT_{719}$ proteins. This band, presently of unknown identity, was not evident for the wild type until 12 h of incubation; in contrast, this band appeared immediately (after 3 min warm-up from 0°C to 37°C) for $TPMT_{460}$ and within 30 min for $TPMT_{719}$. Moreover, this band was observed under denaturing conditions (i.e., stable to SDS/β-mercaptoethanol/100°C heat), suggesting a covalent nature of the conjugates.

*Estimation of TPMT*3 Allele Frequency in Caucasians*

To estimate the allele frequency of *TPMT*3*, PCR-RFLP analysis was used to test for the G460A and A719G mutations in genomic DNA isolated from 22 unrelated Caucasians with RBC TPMT activity indicative of a heterozygous genotype (i.e., 5.0–10 U/ml pRBC) (Weinshilboum and Sladek 1980). The G460A and A719G mutations were detected in 17 of these 22 individuals, indicating that the *TPMT*3* allele was present in ~75% of this heterozygous population. Furthermore, these mutations were not detected in 10 Caucasians with RBC TPMT activity >19 U/ml pRBC, an activity indicative of a homozygous wild-type genotype (Weinshilboum and Sladek 1980).

Discussion

The present investigation has identified the most prevalent mutant allele associated with human TPMT-deficiency in Caucasians (*TPMT*3*), comprising >75% of mutant alleles in the 22 white heterozygote subjects evaluated. We previously identified a mutant TPMT allele (*TPMT*2*) that contains a single nucleotide transversion (G238C) (Krynetski et al. 1995*b*); however, *TPMT*2* comprises a relatively small percentage of *TPMT* mutant alleles. The deficient patient from whom the new mutant allele (*TPMT*3*) was isolated had erythrocyte TPMT protein levels 20–30-fold less than individuals with wild-type phenotypes (fig. 1), indicating that his TPMT-deficiency was associated with low levels of TPMT protein. This association is consistent with previous immunotitration studies demonstrating that the immunoreactive protein of TPMT is correlated with enzymatic activity (Woodson et al. 1982). The residual TPMT protein in our patient may be from his *TPMT*2* allele, which is associated with a 20-fold reduction in TPMT protein when expressed in yeast (data not shown).

Heterologous expression of the *TPMT*3* cDNA in yeast produced TPMT mRNA levels comparable to wild type, indicating that these mutations have no significant impact on transcription in this heterologous expression system. Because the *TPMT*3* cDNA contains two tran-

Table 1

Kinetic Parameters of Substrate (6MP) and Cosubstrate (SAM) for S-Methylation of 6-MP Catalyzed by Human TPMT cDNAs Expressed in Yeast

cDNA Expressed	K_m (μM)	V_{max} (nmol/min/mg TPMT)	V_{max}/K_m (ml/min/mg TPMT)
	6MP		
Wild type	95.3 ± 5.5	260.6 ± 9.8	2.7
TPMT$_{460}$	4396 ± 1367[a]	958.5 ± 187.9[a]	.2
TPMT$_{719}$	182.5 ± 10.1	338.8 ± 13.5	1.9
*TPMT*3*	ND	ND	...
	SAM		
Wild type	6.6 ± 1.1	173.1 ± 14.1	26.2
TPMT$_{460}$	1375 ± 211[a]	704.9 ± 69.7[a]	.51
TPMT$_{719}$	9.5 ± 1.4	226.9 ± 19.1	23.9
*TPMT*3*	ND	ND	...

NOTE.—Kinetic parameters for 6MP were estimated using 1 mM SAM; and parameters for SAM were estimated at 2 mM 6MP. All values are expressed as mean ± SE. ND = Activity not detectable.

[a] Significantly different from wild-type and *TPMT*$_{719}$ ($P < .01$).

sition mutations (G460A and A719G), which differ from the single nucleotide transversion responsible for loss of activity with *TPMT*2* (Krynetski et al. 1995*b*), it was important to determine whether either or both of the *TPMT*3* mutations resulted in loss of activity. Despite comparable TPMT mRNA levels when expressed in yeast (fig. 4), TPMT protein levels were ~400-fold lower for *TPMT*3*, and 4-fold lower for *TPMT*$_{460}$ than for wild type, while *TPMT*$_{719}$ had protein levels comparable to wild type (fig. 4*B*). These data suggest that either the G460A or A719G transition alone has only modest (G460A) or no effect (A719G) on translation in yeast, while the presence of both mutations leads to a marked reduction in TPMT protein. Lower TPMT protein was also observed in erythrocytes from the *TPMT*3* propositus (fig. 1*A*), indicating that the mechanism for loss of function results in lower TPMT protein levels in both yeast and humans. In addition, there was marked instability of TPMT catalytic activity conferred by either point mutation alone, despite the persistence of immunodetectable protein (fig. 5), compared with no detectable activity and markedly lower protein when both mutations were present. It is thus possible that if there are individuals with allelic variants containing only the G460A mutation, they may have low TPMT activity but ample immunodetectable protein. As depicted in figure 5, a higher-molecular-weight protein recognized by an anti-TPMT antibody accumulates during in vitro incubation of recombinant proteins, particularly noteworthy with the mutant cDNAs. While the identity of the 52-kD band is unknown, it could represent the formation of a multiubiquitin chain attached to the unfolded TPMT protein (Ciechanover and Schwartz 1994). Further studies will be required to explore these hypotheses and to provide additional insights into the basis for loss of catalytic activity.

In addition to changes in stability of TPMT activity, the G460A transition was associated with a marked increase in K_m for both 6MP (46-fold) and the cosubstrate SAM (208-fold), such that the intrinsic clearance for 6MP methylation (V_{max}/K_m) was more than 10-fold lower than that of wild-type protein. For comparison, the V_{max}/K_m ratio for heterologously expressed *TPMT*2* (Krynetski et al. 1995*b*) was about fivefold lower than wild-type TPMT (data not shown). Of note, the kinetic parameters for *TPMT*$_{460}$ and *TPMT*$_{719}$ were significantly different, despite similar intrinsic instability for the two mutant proteins.

The *TPMT*3* G460A and A719G mutations were found in genomic DNA isolated from 17 of 22 unrelated white subjects with heterozygous phenotypes, indicating that *TPMT*3* was present in >75% of this selected population. Although 22 unrelated heterozygotes carry the mutant alleles expected in a population of 220 randomly selected subjects (given that *TPMT* heterozygotes comprise ~10% of the white population), larger population studies will be required to precisely establish allele frequencies at the human *TPMT* locus.

We have recently found an unrelated TPMT-deficient patient (McLeod et al. 1993) to be homozygous for the *TPMT*3* allele, by cDNA sequencing and by the PCR-RFLP methods described herein. Thus, TPMT-deficient

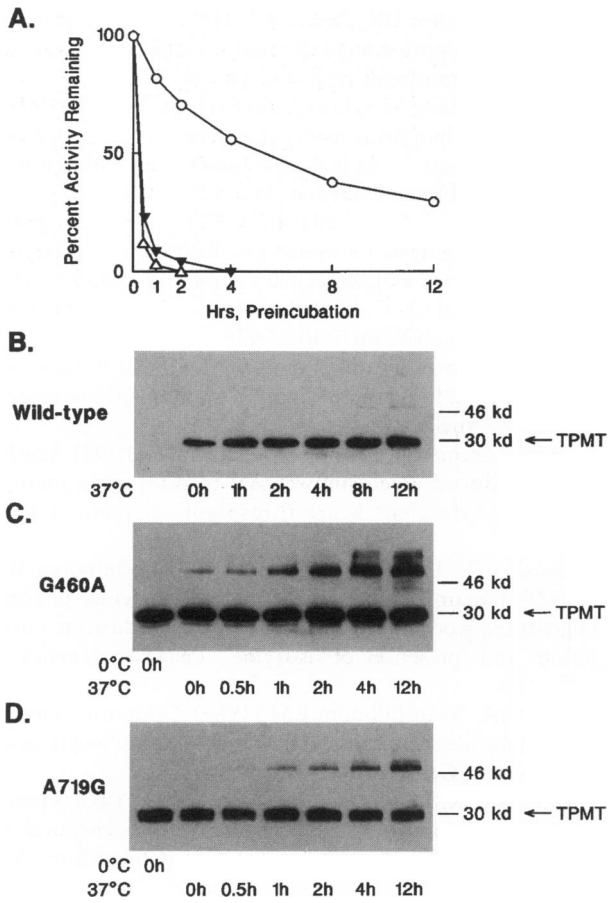

Figure 5 In vitro stabilities of TPMT wild-type and mutant proteins. *A*, TPMT activities expressed as a percentage of those obtained at 37°C, 0 h (i.e., 83.7, 142.5, 88.6 nmol/min/mg TPMT for wild type [○], $TPMT_{460}$ [△], and $TPMT_{719}$ (▼) lysates, respectively; *TPMT*3* had no activity at 0 h. *B–D*, Immunoreactive protein with equal loading of wild-type (*B*), $TPMT_{460}$ (*C*), and $TPMT_{719}$ (*D*) protein.

patients with either *TPMT*2/TPMT*3* or *TPMT*3/TPMT*3* genotypes have now been documented. While additional *TPMT* allelic variants will likely be discovered, the present investigation has identified the major mutant *TPMT* allele associated with TPMT deficiency in Caucasians. It should be recognized that the natural substrate for TPMT has not been identified, and it is not known whether mutations leading to loss of function for S-methylation of thiopurines will also affect catalytic activity for its endogenous substrate(s). Nonetheless, in light of the importance of 6MP for curative therapy of acute lymphoblastic leukemia and the expanding role of azathioprine immunosuppression in organ transplant recipients (Hollander et al. 1995), a DNA-based method to prospectively diagnose TPMT-deficiency offers a clinically important strategy to minimize the risk of potentially life-threatening hematopoietic toxicity in patients treated with these medications.

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